

# The Role of Sphingosine-1-Phosphate in Adipogenesis of Graves' Orbitopathy

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**PURPOSE.** To investigate the action of sphingosine-1-phosphate (S1P) in adipocyte differentiation of orbital fibroblasts and determine its putative role in the pathogenesis of Graves' orbitopathy (GO).

**METHODS.** Primary preadipocyte orbital fibroblast cultures were stimulated for adipogenesis. Real-time PCR was performed to evaluate the expression of S1P receptor mRNA. To evaluate the effect of S1P and S1P receptor blockers (W146 and FTY720) on adipocyte differentiation, cultures were exposed to each receptor blocker for the first 4 days of the differentiation period. Differentiated cells were stained with Oil Red O, and the production of peroxisome proliferator activator gamma (PPAR $\gamma$ ) and CCAAT-enhancer-binding proteins (C/EBP)  $\alpha$  and  $\beta$  were determined by Western blot analysis.

**RESULTS.** Sphingosine-1-phosphate receptor 1, 2, and 3 mRNA expression levels were significantly higher in GO tissue samples than non-GO. Sphingosine-1-phosphate receptor 1 through 5 mRNA expression was significantly increased during the 10 days of adipogenesis. Sphingosine-1-phosphate treatment increased the size and number of adipocytes, and increased the expression of adipogenic transcriptional regulators. Treatment with S1P1 receptor inhibitor (W146) for 4 days after induction of adipogenesis attenuated adipocyte differentiation. Sphingosine-1-phosphate receptor blocker also decreased reactive oxygen species (ROS) production in GO orbital fibroblasts and H<sub>2</sub>O<sub>2</sub>-stimulated HO-1 production in GO orbital fibroblasts. S1P1 receptor inhibitor reduced the number of adipocytes and suppressed the accumulation of lipid droplets induced by 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 2% cigarette smoke extract (CSE) treatment.

**CONCLUSIONS.** Sphingosine-1-phosphate could play a role in orbital adipocyte differentiation of GO. Modulation of S1P actions may provide a therapeutic target for the treatment of GO.

**Keywords:** sphingosine-1-phosphate, Graves' orbitopathy, adipogenesis

Graves' orbitopathy (GO) is an autoimmune disease that occurs in up to 50% of patients with Graves' disease.<sup>1</sup> Severe and potentially blinding orbital inflammation develops in approximately 5% to 6% of these patients.<sup>2</sup> The inflammation causes muscle and soft tissue swelling, which, when progressive, can result in clinical features of proptosis, exposure keratopathy, and compressive optic neuropathy.

Current evidence points to orbital fibroblasts as the target cells in GO and suggests that their normal functions are dysregulated through autoimmune mechanisms.<sup>3</sup> These orbital fibroblasts secrete large quantities of hyaluronan in response to various cytokines, and a subgroup of the orbital fibroblasts then differentiate into mature adipocytes that have increased thyrotropin receptor production.<sup>4,5</sup> These cellular changes lead to enlargement of extraocular muscles and expansion of orbital fat in GO patients.

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that mediates diverse cellular responses such as proliferation, cytoskeletal organization and migration, immune cell trafficking, adherence and tight junction assembly, and differentia-

tion.<sup>6,7</sup> Sphingosine-1-phosphate is formed by the action of sphingosine kinase (SphK), which converts sphingosine and ATP to S1P. Most of its best-characterized actions are mediated through a family of five G protein-coupled receptors, the S1P 1 through 5 receptors, which couple to a variety of G proteins allowing them to mediate many different biologic responses.<sup>6,8</sup>

Among the several interesting actions of S1P, a great deal of attention has been focused on its effects on the process of differentiation. The S1P/S1P1 receptor system plays a crucial role in promoting angiogenesis by regulating differentiation of vascular endothelial cells.<sup>9,10</sup> Sphingosine-1-phosphate also promotes myogenic differentiation of the mouse C2C12 skeletal myocyte cell line,<sup>11</sup> and modulates mouse osteoclast differentiation.<sup>12</sup> Recently, Hashimoto et al.<sup>13</sup> reported that sphingosine kinase and S1P are upregulated during adipogenesis and play a significant role in adipocyte differentiation in mouse 3T3-L1 cells. Also, in 3T3-L1 preadipocytes the expression of 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1), which is known to contribute to adipose tissue differentiation and dysfunction, is robustly induced when cells are treated with



TABLE. Clinical Characteristics of GO and Non-GO Patients in Our Study

	Age	Sex	Surgery Performed	Duration of GO, mo	CAS
GO patients	31	F	Orbital decompression	16	1
	31	M	Orbital decompression	156	2
	29	F	Orbital decompression	50	0
	59	M	Orbital decompression	144	3
	31	F	Orbital decompression	18	1
Non-GO control patients	36	F	Blepharoplasty	n/a	n/a
	42	F	Blepharoplasty	n/a	n/a
	64	F	Orbital wall fracture	n/a	n/a
	55	F	Orbital wall fracture	n/a	n/a
	45	F	Orbital wall fracture	n/a	n/a

S1P.<sup>14,15</sup> Conversely, FTY720, a synthetic analogue of S1P, which acts as a functional antagonist of S1P, downregulates adipogenic differentiation.<sup>16</sup>

To date, no studies have evaluated the expression of S1P receptors and the action of S1P in orbital fibroblasts and its putative role in adipocyte differentiation, one of the most important features of pathogenesis of GO. Here, we hypothesized that S1P could play a role in orbital adipocyte differentiation, and modulation of this bioactive mediator may provide a therapeutic target for the treatment of GO.

## MATERIALS AND METHODS

### Reagents and Chemicals

Sphingosine-1-phosphate (S1P) and FTY720 were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Sphingosine-1-phosphate receptor 1 antagonist, W146 (trifluoroacetate salt) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and gentamycin were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Oil Red O were purchased from Sigma Aldrich, Inc. Anti-peroxisome proliferator activator gamma (PPAR $\gamma$ ), anti-CCAAT-enhancer-binding protein (C/EBP)  $\alpha$ , anti-C/EBP  $\beta$  anti-heme oxygenase (HO)-1, anti-extracellular-regulated kinase (ERK) 1/2, anti-phospho ERK 1/2, anti-phospho-Akt, and anti- $\beta$ -actin antibodies were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Orbital adipose/connective tissue specimens were obtained during the orbital decompression surgery for severe GO ( $n = 5$ ; three women and two men, aged 29–59 years) (Table). The GO patients had not received steroid medication for at least 3 months before surgery, and were euthyroid at the time of surgery. The clinical activity scores (CAS) at the time of tissue harvest were less than four for all patients, indicating inactive inflammatory status, which was based on an original 10-point CAS scale developed by Mourits et al.<sup>17</sup> No patients had been previously treated with orbital radiotherapy. Normal orbital adipose/connective tissue specimens were collected during the course of orbital surgery for other noninflammatory problems from patients with no prior history of thyroid disease or GO and with no clinical evidence of GO ( $n = 5$ ; five women, aged 36–64 years). The institutional review board of Severance Hospital, Yonsei University College of Medicine (Seoul, Korea), approved the study and written informed consent was obtained from all participants. This study followed the tenets of the Declaration of Helsinki, and written informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study.

### Cell Culture and Differentiation Protocol

Tissue explants were minced and placed directly in plastic culture dishes in DMEM:F12 (1:1) containing 20% FBS, penicillin (100 U/mL), and gentamycin (20  $\mu$ g/mL), allowing preadipocyte fibroblasts to proliferate. After fibroblasts had grown out from the explants, monolayers were passaged serially by gently treating with trypsin/EDTA, and cultures were maintained in 80-mm flasks containing DMEM with 10% FBS and antibiotics. Cell cultures were grown in a humidified 5% CO<sub>2</sub> incubator at 37°C. The strains were stored in liquid N<sub>2</sub> until needed, and were used between the second and fifth passage.

After cells reached confluence in 6-well plates, differentiation of adipocytes was initiated by the following protocol. The culture medium was changed to serum-free DMEM supplemented with 33  $\mu$ M biotin, 17  $\mu$ M pantothenic acid, 10  $\mu$ g/mL transferrin, 0.2 nM T3, 1  $\mu$ M insulin (Boehringer-Mannheim, Mannheim, Germany), and 0.2  $\mu$ M carbaprostaglandin (cPGI2; Calbiochem, La Jolla, CA, USA). For the first 4 days, 1  $\mu$ M insulin, 1  $\mu$ M dexamethasone, and 0.1 mM isobutylmethylxanthine were included in the media. The differentiation was continued for 10 days, during which the media was replaced every 2 to 3 days. A PPAR $\gamma$  agonist, rosiglitazone (10  $\mu$ M, Cayman, Ann Arbor, MI, USA), was added from day 1 for further stimulation of adipogenesis.<sup>18</sup> To evaluate the effect of S1P and S1P receptor blockers (W146 and FTY720) on adipocyte differentiation, we exposed cultures to S1P and S1P receptor blockers for the first 4 days of the differentiation period.

### Real-Time PCR

Total RNA (1  $\mu$ g) was isolated and reverse transcribed into complementary DNA according to the manufacturer's instructions (#74104; Qiagen, Valencia, CA, USA). The resulting cDNA was amplified using an ABI StepOnePlus real-time PCR thermocycler (Applied Biosystems, Carlsbad, CA, USA) using TaqMan universal PCR master mix and recommended PCR conditions to assess gene transcript levels quantitatively in the cell samples. All PCR reactions were performed in triplicate. Primer sequences are as follows: human S1P receptor 1, 5'-TGC GGG AAG GGA GTA TGT TT-3' (forward) and 5'-CCA TCC CCA CCA CAC TCA AC-3' (reverse); human S1P receptor 2, 5'-GCC TCT CTA CGC CAA GCA TTA-3' (forward) and 5'-TTG AGC GGA CCA CGC AGT A-3' (reverse); human S1P receptor 3, 5'-TGA TTG TGG TGA GCG TCT TCA-3' (forward) and 5'-GGC CAC ATC AAT GAG GAA GAG-3' (reverse); human S1P receptor 4, 5'-CGG CAC AGC CTC CTC ATT GTC-3' (forward) and 5'-ACA GAC CGA TGC AGC CAT ACA CAC-3' (reverse); for human S1P receptor 5, 5'-CGT CCT GCA TTA CAA CTA CAC C-3' (forward) and 5'-GGC CAC ATC AAT GAG GAA GAG-3' (reverse); and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) 5'-GCC AAG GTC ATC CAT GAC AAC-3' (forward) and

5'-GTC CAC CAC CCT GTT GCT GTA-3' (reverse). All samples were normalized to the GAPDH values and the results are expressed as the relative fold change of threshold cycle (Ct) value relative to the control group using the  $2^{-\Delta\Delta C_t}$  method.<sup>19</sup> Amplification bands were quantified by densitometry and normalized against corresponding GAPDH bands to control for PCR variability.

### Oil Red O Staining

Cells were stained with Oil Red O as described by Green and Kehinde.<sup>20</sup> A 0.5% (wt/vol) stock solution of Oil Red O in isopropanol was prepared. For the working solution, 6 mL of the stock solution was mixed with 4 mL distilled water, left for 1 hour at room temperature, and then filtered through a 0.2- $\mu$ m filter. Cells were washed twice with 1x PBS, fixed with 3.7% (wt/vol) formalin in PBS for 1 hour at 4°C, and stained with 300  $\mu$ L Oil Red O working solution for 1 hour at room temperature. The cells were washed with distilled water before being visualized using an Axio Vert (Carl Zeiss, Jena, Germany) light microscope and photographed at  $\times 40$ ,  $\times 100$ , and  $\times 200$  magnification with an Olympus BX60 light microscope (Olympus, Melville, NY, USA). To measure lipid accumulation, cell-bound Oil Red O was solubilized with 100% isopropanol, and the optical density of the solution was measured using a spectrophotometer at 490 nm. Experiments for the quantitative assessment of adipogenic differentiation were performed in triplicate in cells from different donors, and the results were normalized to the absorbance of untreated differentiated control cells.

### Western Blot Assays

Differentiated cells were washed with ice-cold PBS and lysed on ice for 30 minutes in cell lysis buffer consisting of 20 mM HEPES (pH 7.2), 10% (vol/vol) glycerol, 10 mM  $\text{Na}_3\text{VO}_4$ , 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1  $\mu$ g/mL leupeptin, 1  $\mu$ g/mL pepstatin, and 1% (vol/vol) Triton X-100. Reagents were obtained from Sigma-Aldrich Corp. Lysates were centrifuged for 15 minutes at 21,760g and cell homogenate fractions were stored at  $-70^\circ\text{C}$  before use. Protein concentrations in supernatant fractions were determined by the Bradford assay (BioRad, Hercules, CA, USA). Equal amounts of protein (50  $\mu$ g) were boiled in sample buffer and resolved by SDS-PAGE in 10% (wt/vol) gels. The separated proteins were transferred to polyvinylidene fluoride membranes (Immobilon, Millipore, Billerica, MA, USA), probed overnight with primary antibodies in Tris Buffer Saline Tween 20, and washed three times with Tris Buffer Saline Tween 20. Immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibody, and the bound peroxidase was visualized using an enhanced chemiluminescence kit and exposure to X-ray film (GE healthcare, Piscataway, NJ, USA). The relative amount of each immunoreactive band was quantified by densitometry and normalized to  $\beta$ -actin in the same sample.

### Reactive Oxygen Species (ROS) Measurement

Reactive oxygen species release was determined using the oxidant-sensitive fluorescent probe 5-(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate ( $\text{H}_2\text{DCFDA}$ ; Invitrogen, Eugene, OR, USA) as previously described.<sup>21</sup>  $\text{H}_2\text{DCFDA}$  is deacetylated intracellularly by esterase, forming  $\text{H}_2\text{DCF}$  which is oxidized by ROS to 2',7'-dichlorofluorescein (DCF), a highly fluorescent compound. The cells were seeded at a density of  $5 \times 10^5$  cells per well in six-well plates to a total final volume of 2 mL. The cells were pretreated with 10  $\mu$ M W146 or 1  $\mu$ M FTY720 for 1 hour in the presence or absence of 2% cigarette smoke extract

(CSE) or 200  $\mu$ M  $\text{H}_2\text{O}_2$  for 1 hour. The culture medium was then removed, the cells were washed with PBS, and incubated with 10  $\mu$ M  $\text{H}_2\text{DCFDA}$  at  $37^\circ\text{C}$ . The cells were then trypsinized, washed, and resuspended in PBS. Thereafter, fluorescence intensity was measured using a flow cytometer (ELITE flow cytometer; Coulter Cytometry, Inc., Hialeah, FL, USA).

### Preparation of CSE

Cigarette smoke extract was prepared by bubbling smoke from two commercially available filtered cigarettes (Marlboro 20 class A cigarettes, containing 8.0 mg of tar and 0.7 mg of nicotine; Philip Morris Korea, Inc., Seoul, Korea) through 20 mL prewarmed serum-free DMEM/F12 (1:1) at a rate of one cigarette per 2 minute, as described previously.<sup>21</sup> The pH of the CSE was adjusted to 7.4 and the CSE was filter sterilized through a 0.2- $\mu$ m filter (Sartorius Stedim Biotech, Gottingen, Germany), which was then standardized by measuring its absorbance (optical density =  $0.65 \pm 0.05$  at 320 nm). The spectrographic pattern of absorbance at 320 nm showed very little variation between different CSE preparations. The CSE was freshly prepared within 1 hour of each experiment and diluted with culture medium, adjusted to a pH of 7.4, and filter sterilized as described for 10% CSE.

### Statistical Analysis

All experiments were performed at least three times and samples were assayed in duplicate each time. For statistical analysis of semiquantitative PCR assays and Western blots, the mean value and SD was calculated for normalized measurements of each mRNA or protein from multiple ( $\geq 3$ ) samples harvested from different individuals. Data between or within cell groups at different drug concentrations and incubation times were analyzed by the *t*-test or ANOVA test and Bonferroni method as a post hoc test using the SPSS program for Windows, version 16 (SPSS, Chicago, IL, USA). A *P* value less than 0.05 was considered significant.

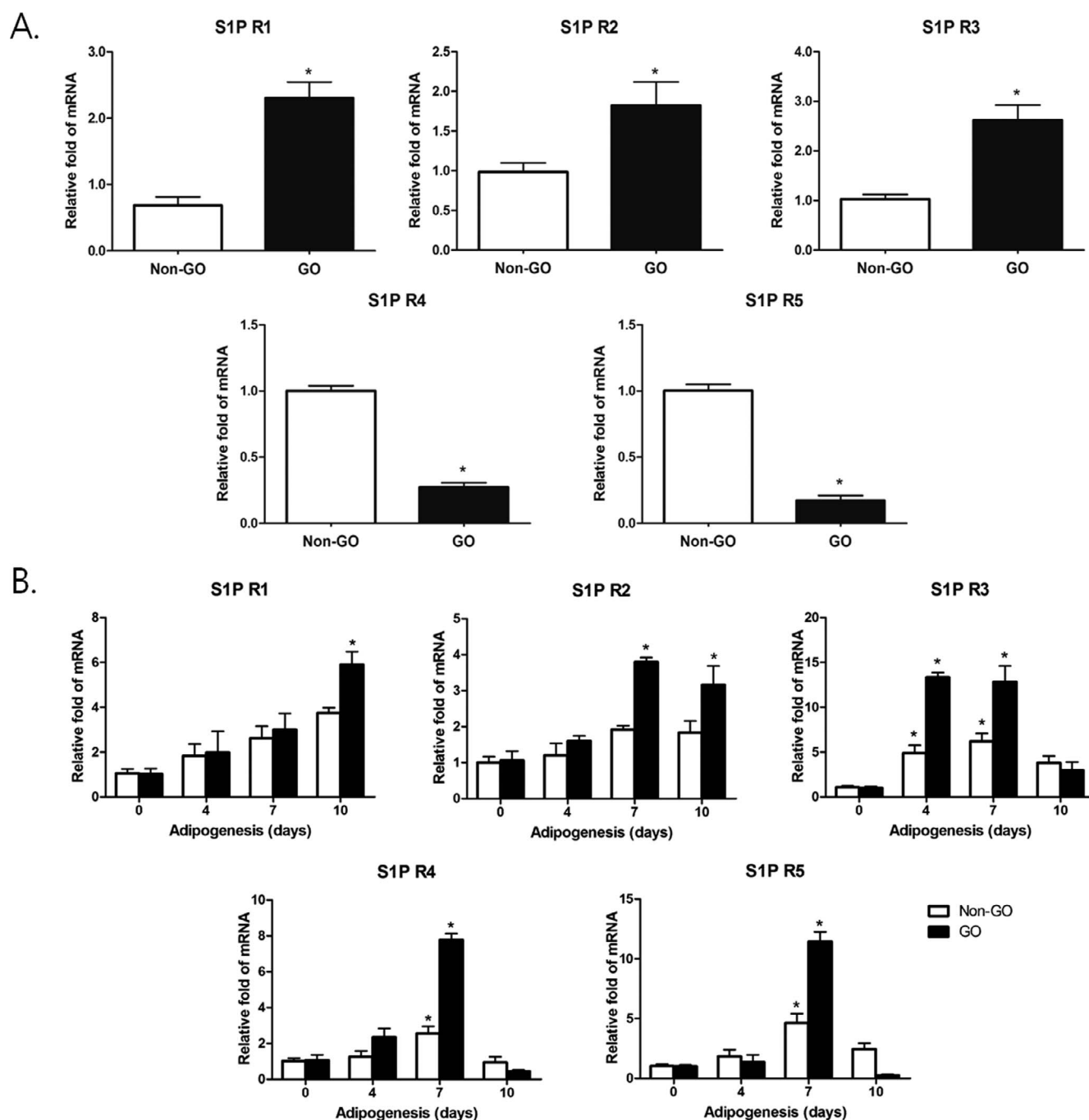
## RESULTS

### S1P Receptor 1 Through 5 mRNA Expression in Orbital Fibroblasts

To compare the expression of S1P receptor mRNAs in GO and non-GO orbital tissues, we performed relative quantification of mRNA for each receptor by RT-PCR using whole orbital tissue explants obtained from five GO and five non-GO control patients. Sphingosine-1-phosphate 1, S1P2, and S1P3 mRNA were expressed at relatively high levels in GO orbital tissues relative to non-GO tissues ( $^*P < 0.05$ ); however, S1P receptor 4 and 5 gene levels were lower in GO tissues (Fig. 1A). We also measured S1P receptor 1 through 5 mRNA expression on days 0, 4, 7, and 10 of adipocyte differentiation of GO fibroblasts ( $n = 3$ ) and non-GO fibroblast ( $n = 3$ ) cultures. All S1P receptor types were significantly increased during the adipogenesis of GO fibroblasts. Receptor gene expression seemed to increase over time and was usually highest on day 7 (Fig. 1B). The receptor gene expression pattern during adipogenesis of non-GO fibroblast was similar to that of GO, yet the expression levels were comparatively low in non-GO cultures.

### Effect of S1P on GO Orbital Fibroblast Adipogenesis

Confluent orbital fibroblasts from GO patients ( $n = 3$ ) were subjected to an adipocyte differentiation protocol for 10 days.

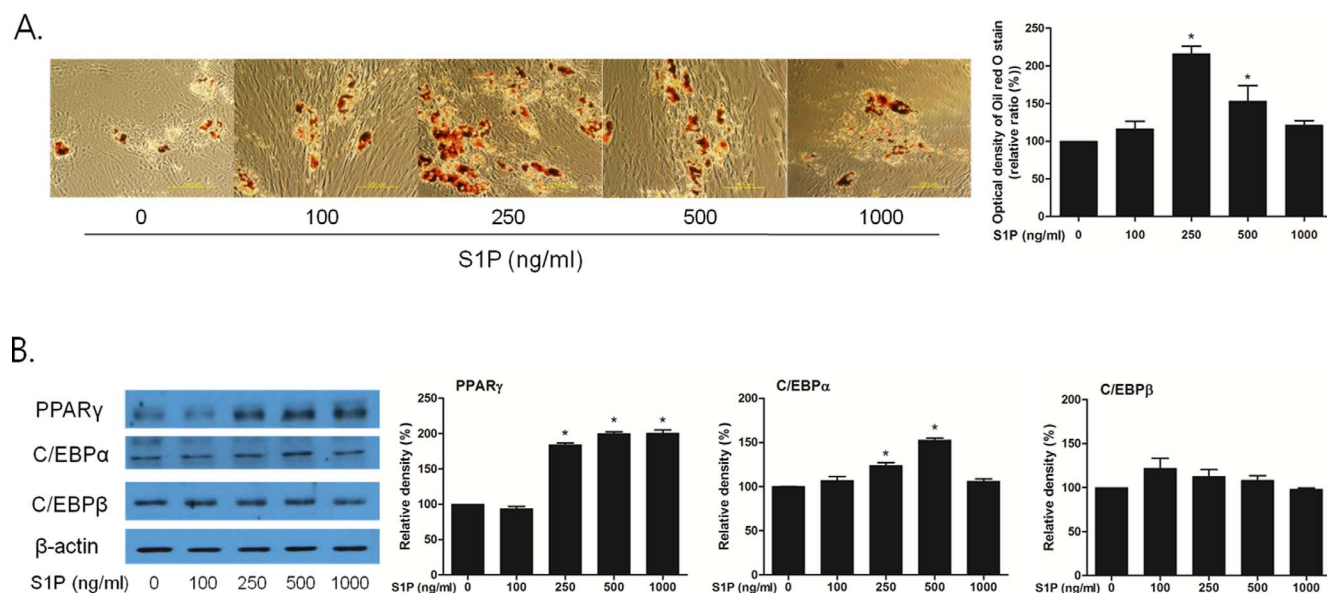


**FIGURE 1.** Expression of S1P receptor mRNAs in GO and non-GO orbital tissues, and differentiating Graves' orbital fibroblast cultures. (A) Comparison of S1P receptor 1 through 5 mRNA expression using real-time PCR between non-GO controls ( $n = 5$ ) and GO ( $n = 5$ ) tissue samples. Sphingosine-1-phosphate receptor 1, 2, and 3 mRNA expression levels were all significantly higher in GO tissues samples relative to non-GO tissues ( $*P < 0.05$ ). (B) Sphingosine-1-phosphate receptor 1 through 5 mRNA expression was significantly increased during the 10 days of adipogenesis in orbital fibroblast cultures from different non-GO ( $n = 3$ , white columns) and GO orbital tissues ( $n = 3$ , black columns). The data in the columns are the mean relative fold of mRNA  $\pm$  SD of three experiments ( $*P < 0.05$  versus control undifferentiated cells).

The cells were examined under light microscopy and then stained with Oil Red O. As previously reported, the orbital fibroblasts lost their stellate fibroblastic appearance and converted to a spherical adipocytic shape under the control adipogenic conditions, and a fraction of these cells accumulated small lipid droplets.<sup>22</sup> To examine whether treating with S1P had any effect on adipogenesis, different S1P concentrations (0–1000 ng/mL) were added to the adipogenic medium for 4 days during the differentiation period, being replaced whenever the

medium was replaced. High-power ( $\times 200$ ) microscopic examination of Oil Red O staining showed that S1P treatment increased the size and number of adipocytes and increased the accumulation of lipid droplets with the 250 and 500 ng/mL S1P treatments showing a prominent increase (Fig. 2A). Western blot analysis was performed to investigate whether S1P affects the production of adipogenic transcription factors during adipogenesis. Each experiment was performed in three GO cells from different patient samples and was assayed in duplicate





**FIGURE 2.** Effect of S1P on adipogenesis of Graves' orbital fibroblasts. (A) Different S1P concentrations (100, 250, 500, or 1000 ng/mL) were used to treat orbital fibroblasts during the first 4 days of adipogenic differentiation. Cells were stained with Oil Red O and examined microscopically ( $\times 200$ ). Adipogenesis was increased by S1P treatment compared with the control. The increased adipocyte differentiation was prominent on Oil Red O staining when exposed to 250 and 500 ng/mL S1P treatments. The optical density of stained cell lysates also showed significantly increased absorbance at 490 nm with 250 and 500 ng/mL S1P treatment ( $*P < 0.05$  versus untreated control). (B) Western blot analysis for PPAR $\gamma$ , C/EBP $\alpha$ , and C/EBP $\beta$  protein production after 10 days of adipogenic differentiation. The experiments were performed in triplicate with cells from three different donors. Quantification of the PPAR $\gamma$ , C/EBP $\alpha$ , and C/EBP $\beta$  levels by densitometry were normalized to the level of  $\beta$ -actin in the same sample. The data in the columns are the mean relative density ratios (%)  $\pm$  SD of three experiments ( $*P < 0.05$  versus untreated differentiated control cells).

per each cell sample. As shown in Figure 2B, PPAR $\gamma$  and C/EBP $\alpha$  were enhanced in cells treated with the 250 and 500 ng/mL S1P; however, C/EBP $\beta$  did not increase significantly.

### Effect of the W146 S1P Receptor 1 Blocker on Adipogenesis in GO Orbital Fibroblasts

S1P1 receptor inhibitor, a S1P receptor 1 blocker, was added at different concentrations to the adipogenic medium for the first 4 days during the differentiation period to examine whether W146 has a suppressive effect on adipogenesis. S1P1 receptor inhibitor reduced the number of adipocytes and suppressed the accumulation of lipid droplets, as visualized by microscopic examination after Oil Red O staining (Fig. 3A). The optical density of stained cell lysates was also measured to evaluate adipocyte differentiation quantitatively. At 10 and 20  $\mu$ M concentrations, W146 treated cells showed significantly decreased absorbance at 490 nm ( $P < 0.05$ ). Western blot analyses were then performed to investigate whether W146 affects the production of the adipogenic transcription factors PPAR $\gamma$ , C/EBP $\alpha$ , and C/EBP $\beta$  during adipogenesis. PPAR $\gamma$  and C/EBP $\alpha$  proteins were significantly attenuated by 20  $\mu$ M W146 treatment (Fig. 3B). Each experiment was performed in three GO cells from different patient samples and was assayed in duplicate per each cell sample.

### Expression of S1P Receptors 1 Through 5 Under Oxidative Stress Conditions

We measured S1P receptor 1 through 5 mRNA expression under oxidative stress conditions. Orbital fibroblasts of GO ( $n = 3$ ) and non-GO ( $n = 3$ ) patients were exposed to 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 2% CSE for 0, 1, 3, and 6 hours. All S1P receptor types were significantly increased under the oxidative stress

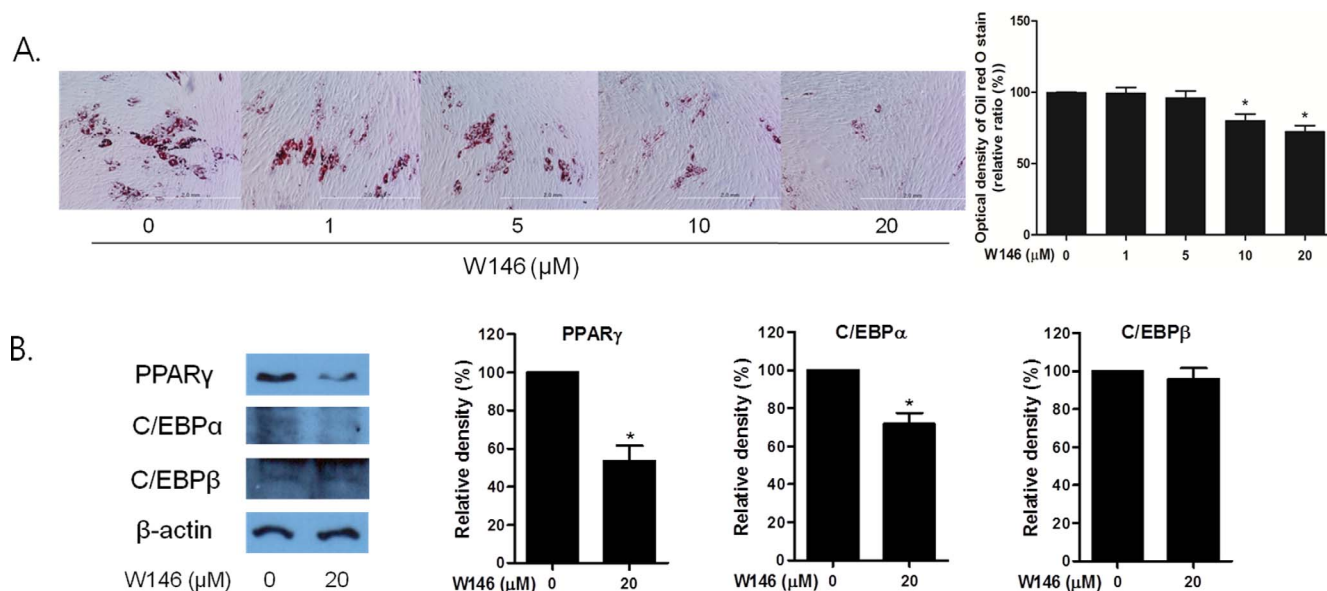
conditions induced with H<sub>2</sub>O<sub>2</sub> by 6 hours (Fig. 4A). However, the expression levels were comparatively low in non-GO cultures. Two percent of CSE also induced expression of S1P receptor 1, 2, 3, and 5 mRNA in GO fibroblast cultures by 6 hours, yet the elevation levels in non-GO cultures were not statistically significant (Fig. 4B).

### S1P Receptor Blockers Inhibit HO-1 Production and ROS Generation in GO Orbital Fibroblasts

Production of the antioxidant enzyme hemeoxygenase-1 (HO-1) in GO fibroblasts was significantly upregulated by treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 4 hours. Pretreatment with S1P receptor antagonists 10  $\mu$ M W146 or 1  $\mu$ M FTY720 for 1 hour significantly suppressed HO-1 production in cells stimulated with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Fig. 4C). In addition, we examined whether S1P receptor blockers decrease ROS production in orbital fibroblasts from GO. When pretreated with or without 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 1 hour, treatment of orbital fibroblasts with the 10  $\mu$ M W146 or 1  $\mu$ M FTY720 significantly decreased ROS levels detected by the ROS-sensitive fluorescent probe, DCFDA (Fig. 4D). Each experiment was performed in three GO cells from different patient samples and was assayed in duplicate per each cell sample.

### Signaling Pathway of S1P Action Under H<sub>2</sub>O<sub>2</sub>-Induced Oxidative Stress

To investigate the molecular mechanism of S1P in GO orbital fibroblasts under oxidative stress conditions, we observed the effect of S1P on ERK1/2 activation. Sphingosine-1-phosphate at 500 ng/mL provoked a rapid and relevant activation of ERK 1/2 in GO cells, whose phosphorylation was maximal at 30 minutes and declined to basal levels at 180 minutes (Fig. 5A).



**FIGURE 3.** Adipogenesis after S1P receptor blocker treatment. **(A)** Treatment with the S1P receptor 1 inhibitor W146 for 4 days after induction of adipogenesis was shown to attenuate adipocyte differentiation as shown with microscopic examination ( $\times 100$ ) of Oil Red O staining. The optical density of stained cell lysates was also measured to evaluate adipocyte differentiation quantitatively. The W146 treated cells at 10 and 20  $\mu\text{M}$  concentrations showed significantly decreased absorbance at 490 nm ( $*P < 0.05$  versus untreated control). **(B)** A representative Western blot of PPAR $\gamma$ , C/EBP $\alpha$ , and C/EBP $\beta$  protein production after 20  $\mu\text{M}$  W146 treatment. PPAR $\gamma$  and C/EBP $\alpha$  proteins were significantly attenuated by 20  $\mu\text{M}$  W146 treatment; however, C/EBP $\beta$  was not significantly affected. Quantification of PPAR $\gamma$ , C/EBP $\alpha$ , and C/EBP $\beta$  protein levels by densitometry, normalized to the level of  $\beta$ -actin in the same sample, is shown. The data in the columns are the mean relative density ratios (%)  $\pm$  SD of three experiments ( $*P < 0.05$  versus untreated differentiated control cells).

Treatment with PD98059 significantly blocked the S1P-provoked phosphorylation of ERK 1/2 (Fig. 5B). The phosphorylation of ERK 1/2 was also strongly enhanced at 30- to 60-minutes when challenged with 200  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (Fig. 5C). When cells were pretreated with W146 and FTY720,  $\text{H}_2\text{O}_2$ -induced phospho-ERK was significantly inhibited (Fig. 5D). Each experiment was performed in three GO cells from different patient samples and was assayed in duplicate per each cell sample.

### Effects of W146 on $\text{H}_2\text{O}_2$ - or CSE-Stimulated Adipogenesis of GO Orbital Fibroblasts

To determine the additive effect of W146 on adipogenesis and against oxidative stress, we stimulated adipogenesis by addition of 10  $\mu\text{M}$   $\text{H}_2\text{O}_2$  or 2% CSE along with W146 for the first 3 days of differentiation. As previously reported by Yoon et al.,<sup>23</sup> the addition of 10  $\mu\text{M}$   $\text{H}_2\text{O}_2$  or 2% CSE significantly increased adipogenesis compared with that observed under control conditions, which was assessed with light microscopy ( $\times 40$ ) after Oil Red O staining (Fig. 6). When W146 (1–20  $\mu\text{M}$ ) was added to the adipogenic medium for the first 4 days during the differentiation period, W146 reduced the number of adipocytes and suppressed the accumulation of lipid droplets induced by 10  $\mu\text{M}$   $\text{H}_2\text{O}_2$  or 2% CSE treatment (Fig. 6). The optical density of stained cell lysates was also measured to evaluate adipocyte differentiation quantitatively. The W146-treated cells showed a decreased absorbance at 490 nm ( $*P < 0.05$ , in contrast to cells treated with 10  $\mu\text{M}$   $\text{H}_2\text{O}_2$  or 2% CSE only).

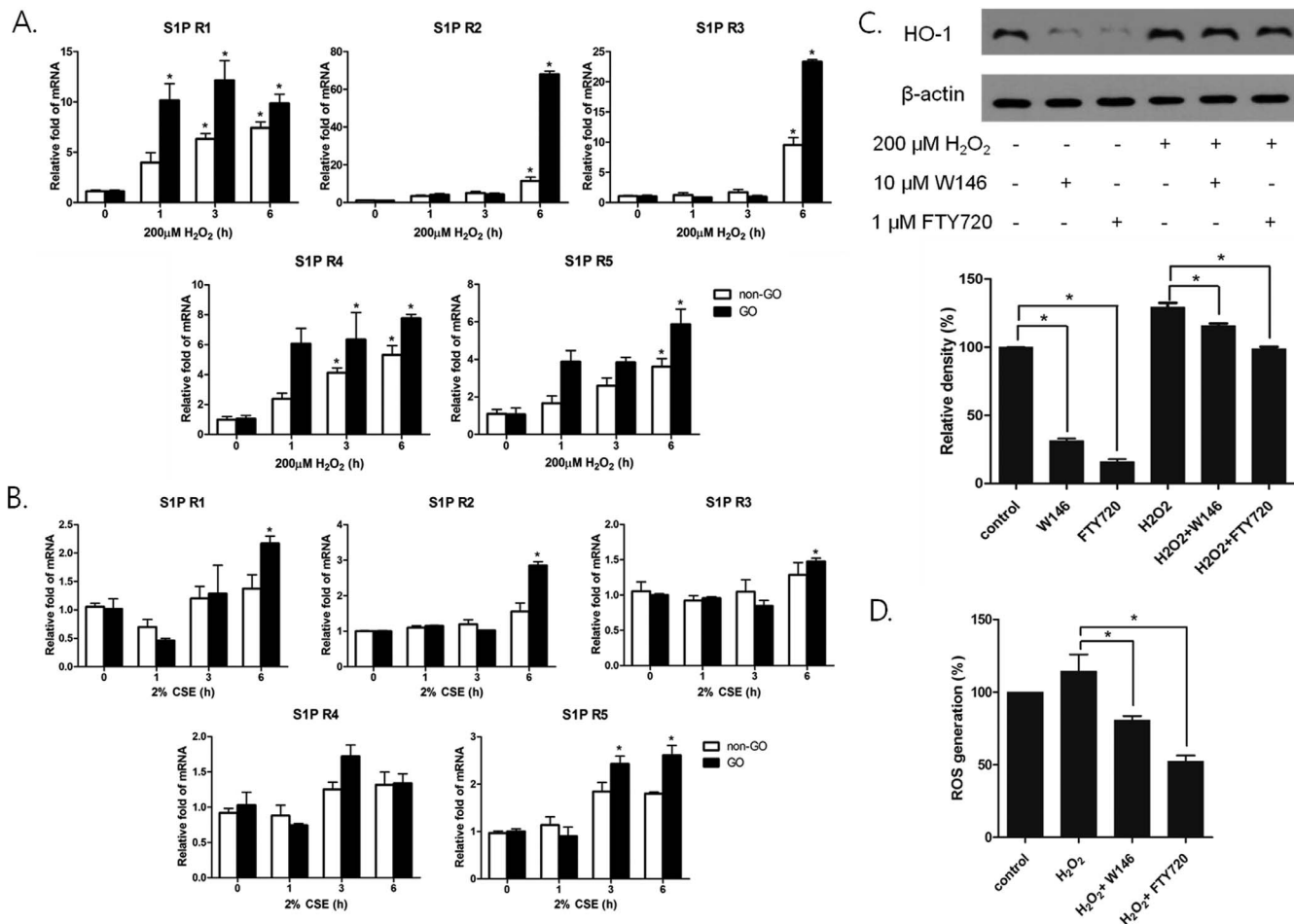
### DISCUSSION

Sphingosine-1-phosphate is involved in many biological activities including cell proliferation, survival, migration, cytoskel-

etal organization, and morphogenesis.<sup>6,24</sup> In this study, we focused on the role of S1P in GO adipogenesis. Previous research with 3T3-L1 cells showed that S1P plays a significant role during adipocyte differentiation.<sup>13</sup> FTY720, a functional antagonist of S1P, was found to downregulate the adipogenesis of 3T3-L1 preadipocytes.<sup>16</sup>

In our study, we first identified the distribution of S1P receptor isoforms in GO tissues in comparison with non-GO orbital fat tissue, and which showed higher level of S1P receptor 1, 2, and 3 in GO than in non-GO. Sphingosine-1-phosphate receptor mRNA expression was significantly increased in GO cells overtime during adipogenic differentiation. The receptor gene expression patterns of non-GO orbital fibroblasts during adipogenesis and under oxidative conditions were similar to that of GO, yet the expression levels were comparatively low in non-GO cultures. Therefore, we could assume that the action of S1P and S1P receptor blocker in adipogenesis and oxidative stress condition would be specific in GO.

Interestingly, as shown in Figure 1B, all S1P receptor mRNA expression was increased during the adipogenic differentiation, however S1P receptor 2 to approximately 5 mRNA decreased from day 7 to day 10. We consider it to be due to the maturation of preadipocyte to adipocyte at the end of adipogenic differentiation. Sphingosine-1-phosphate may affect adipogenesis during the active differentiating phase of adipogenesis. There is a similar report that activity of sphingosine kinase, which are responsible for S1P biosynthesis from sphingosine, was increased in early adipogenic differentiation and decreased after day 6 of adipogenesis and S1P receptor 1 to approximately 3 mRNA levels were high in preadipocyte state and declined after full differentiation.<sup>25</sup> Exogenous S1P treatment enhanced adipocyte differentiation as shown in Oil red O stain, and also increased the expression



**FIGURE 4.** Effect of oxidative stress on S1P receptors and the S1P receptor blockers on ROS production. (A) After treatment with 200  $\mu$ M  $H_2O_2$  for 0, 1, 3, or 6 hours, S1P receptor 1 through 5 gene expression was increased in both non-GO ( $n = 3$ , white column) and GO ( $n = 3$ , black column) orbital fibroblasts ( $*P < 0.05$ ). A significant increase was detected after a 6-hour  $H_2O_2$  treatment. (B) After treatment with 2% CSE for 0, 1, 3, and 6 hours, S1P receptor 1 through 5 gene expression showed a significant increase in GO orbital fibroblasts (black column;  $*P < 0.05$  versus control). However, the increase was not statistically significant in non-GO cultures (white column). (C) Effects of the W146 and FTY720 S1P receptor blockers on  $H_2O_2$ -stimulated HO-1 production in preadipocyte orbital fibroblasts, analyzed with Western blotting. Orbital fibroblasts ( $5 \times 10^5$ ) from GO patients ( $n = 3$ ) were pretreated with 10  $\mu$ M W146 or 1  $\mu$ M FTY720 for 1 hour, and then stimulated with 200  $\mu$ M  $H_2O_2$  for 4 hours to determine the effect of the S1P receptor antagonists on  $H_2O_2$ -stimulated HO-1 protein production. Quantification of HO-1 by densitometry, normalized to the level of  $\beta$ -actin in the same sample, is shown. (D) GO orbital fibroblasts were incubated with  $H_2DCFDA$  10  $\mu$ M for 24 hours with 10  $\mu$ M W146, and 1  $\mu$ M FTY720 in the presence or absence of 200  $\mu$ M  $H_2O_2$  for 1 hour and the fluorescence intensities were analyzed using FACS W146 and FTY720 decreased ROS levels significantly in orbital fibroblasts from GO. Results are expressed as a percentage of untreated control values presented as the mean  $\pm$  SD of three separate experiments ( $*P < 0.05$  versus cells treated with 200  $\mu$ M  $H_2O_2$  only).

of adipogenic transcriptional markers. Conversely, adipogenesis was significantly attenuated by blockade of S1P1 receptor.

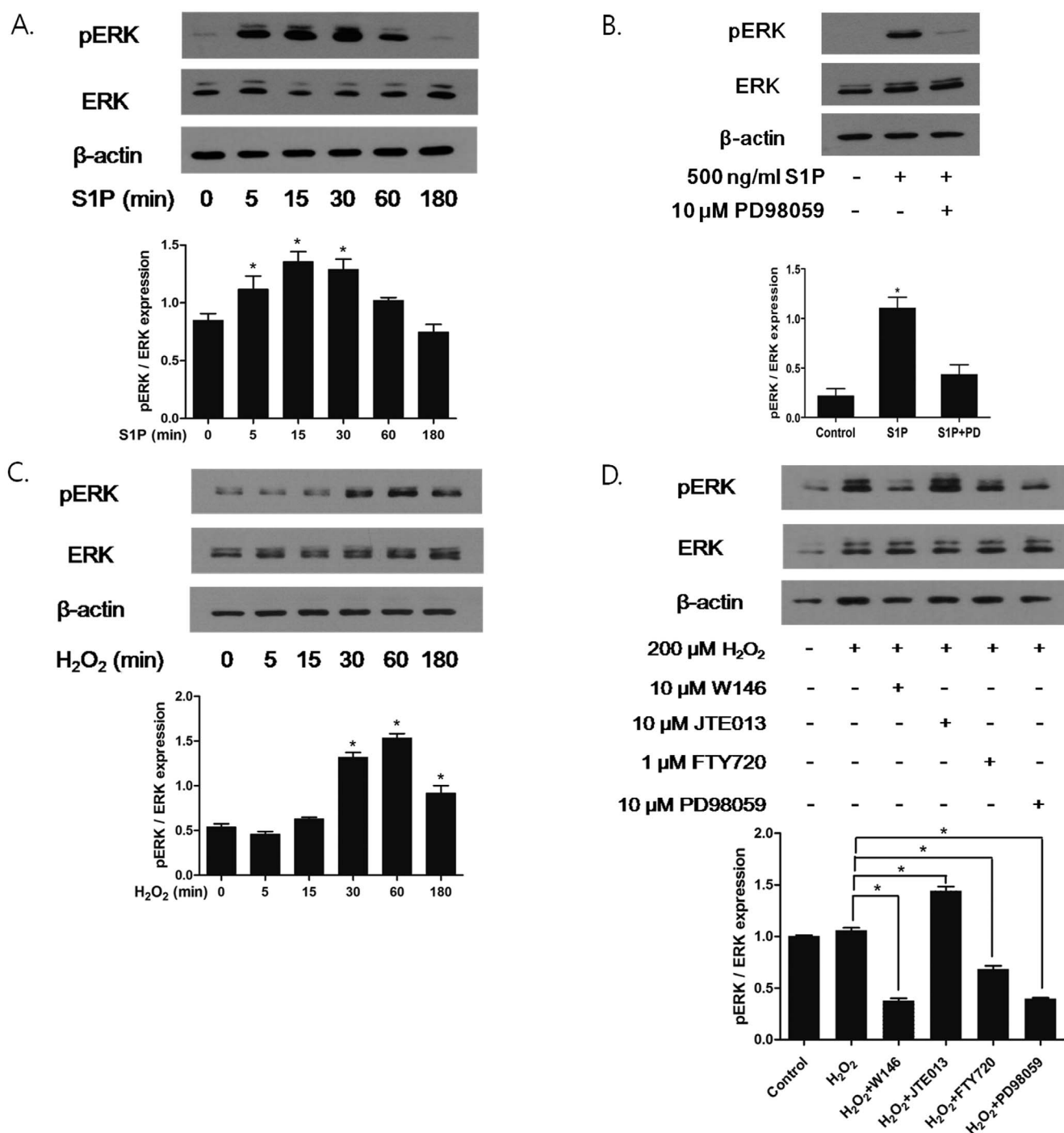
In this study, we found that W146 suppressed adipogenesis in orbital fibroblasts and reduced the protein levels of adipogenesis-related transcriptional factors, PPAR $\gamma$  and C/EBP $\alpha$ . PPAR $\gamma$  and C/EBP transcription factors are produced at distinct phases during adipogenesis, which have been shown to play important roles. Sphingosine-1-phosphate 1 receptor antagonist, W146 that exhibits no agonist or antagonist activity at S1P $_2$ , S1P $_3$ , or S1P $_5$  receptors is known to cause in vivo skin capillary leakage in murine lung and skin as well as inhibition of S1P $_1$  agonist-induced lymphocyte sequestration.<sup>26</sup> Our data suggest that W146 exert antiadipogenic effect by suppressing these adipogenic transcription factors.

Oxidative stress plays an important role in the pathogenesis of GO.<sup>27,28</sup> Oxygen free radicals contribute to the proliferation of orbital fibroblasts and glycosaminoglycan production.<sup>29</sup> Smoking is the strongest known environmental factor, which

stimulates the development and deterioration of GO by enhancing ROS generation and adipogenesis.<sup>30</sup>  $H_2O_2$ -induced elevation of superoxide anions can be abolished by antioxidant treatment, however.<sup>31</sup> Recently, selenium (an antioxidant) was successfully applied in patients with mild GO in a large, multicenter, randomized, placebo-controlled trial in Europe.<sup>32</sup> Antioxidants may exert their actions through antioxidative or anti-inflammatory effects.

There is a growing evidence that the sphingolipid rheostat also plays a pivotal role in cellular response to oxidative stress, determining whether cells survive or proceed to apoptosis.<sup>33,34</sup> Moderate levels of oxidative stress activate Sphingosine kinase 1 (SphK1), an enzyme which phosphorylates sphingosine to S1P, making a shift in the ceramide-S1P balance toward S1P, and that suppress apoptosis and maintain cell survival and proliferation. However, excessive oxidative stress leads to SphK1 degradation, which decreases S1P, shifting the balance of the rheostat toward apoptosis. As shown in this study, S1P



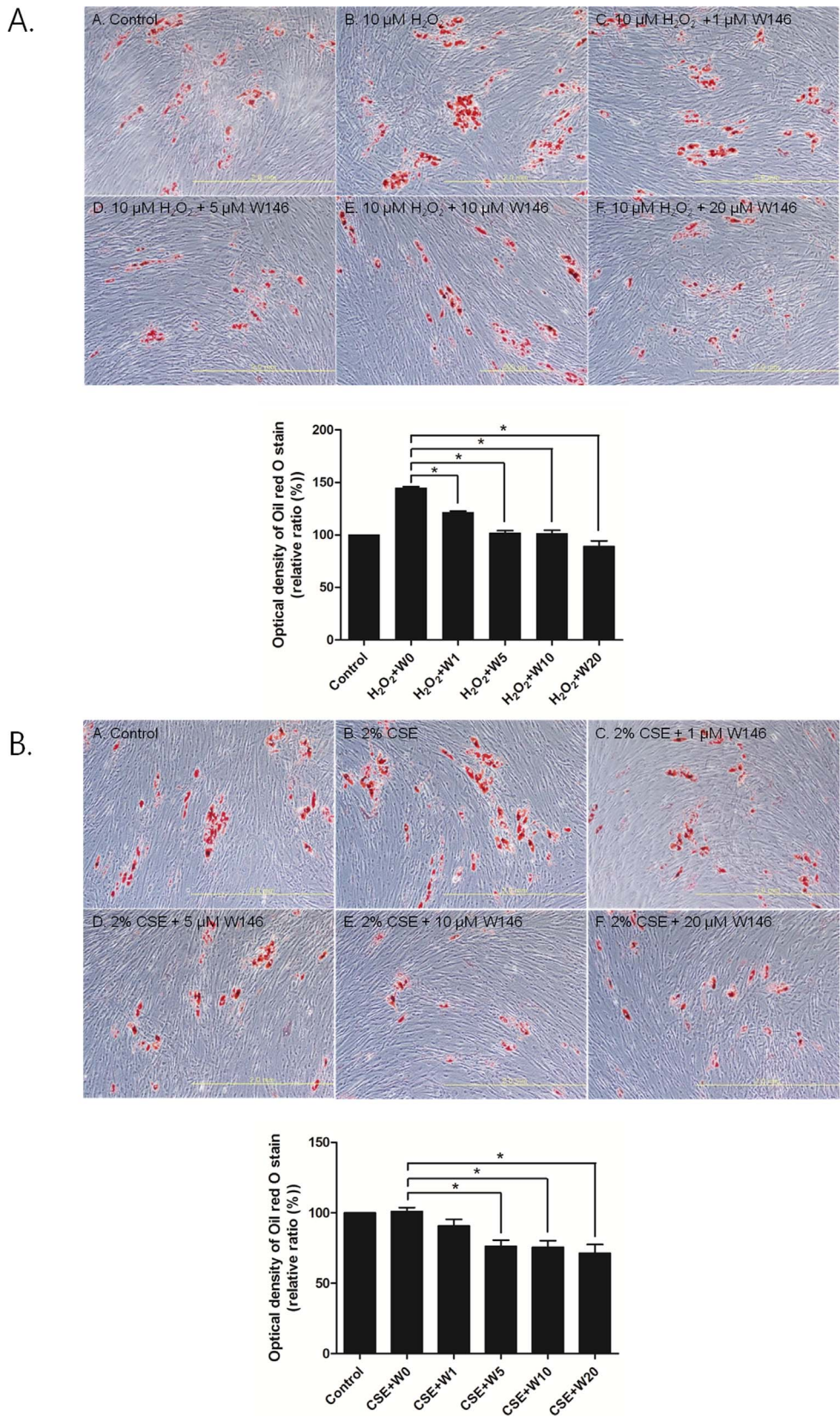


**FIGURE 5.** Effect of S1P on activation of ERK signal in Graves' orbital fibroblasts. **(A)** Treatment with 500 ng/mL S1P provoked activation of ERK 1/2, phosphorylation of which was maximal at 30 minutes and declined to basal levels at 180 minutes ( $^*P < 0.05$  versus untreated control). **(B)** Treatment with PD98059 significantly blocked the S1P-provoked phosphorylation of ERK 1/2. **(C)** The phosphorylation of ERK 1/2 was strongly enhanced at 30- to 60-minute challenge with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub>. **(D)** When cells were pretreated with 10  $\mu$ M W146 and 1  $\mu$ M FTY720, H<sub>2</sub>O<sub>2</sub>-induced phospho-ERK activation was significantly inhibited. The data in the columns are the mean relative density fold  $\pm$  SD of three experiments ( $^*P < 0.05$  versus untreated control differentiated cells).

seems to play a role in the response to oxidative stress in GO orbital fibroblasts. In our study, W146 and FTY720 significantly inhibited H<sub>2</sub>O<sub>2</sub>-induced antioxidant enzyme HO-1 production and ROS generation in GO orbital fibroblasts (Fig. 4). The induction of HO-1 is considered a part of the protective response to oxidative stress, as an active defense mechanism.<sup>23</sup>

Antioxidant enzyme hemeoxygenase-1 was upregulated in orbital fibroblasts by oxidative stress and pretreatment with S1P receptor antagonists reduced the production of HO-1. This reduction in HO-1 seems to be a consequence of the reduction in intracellular ROS induced by S1P receptor antagonist activity.





**FIGURE 6.** Effects of W146 on  $H_2O_2$ -stimulated adipogenesis. Cells from GO patients ( $n = 3$ ) were treated with 10  $\mu$ M  $H_2O_2$  (**A**) or 2% CSE (**B**) for the first 3 days of the 10-day adipogenesis period in adipogenic medium containing 10  $\mu$ M rosiglitazone. To determine the suppressive effect of W146 on adipogenesis, W146 (1–20  $\mu$ M) was also added for the first 4 days of differentiation. The cells were stained with Oil Red O and examined microscopically ( $\times 40$ ). The experiments were performed in triplicate with cells from three different GO donors, and representative images are shown. The optical density of stained cell lysates was also measured ( $^*P < 0.05$ , in contrast to cells treated with 10  $\mu$ M  $H_2O_2$  only).

We also identified one signaling cascade that appears to control the action of S1P under oxidative stress. Our results suggest that H<sub>2</sub>O<sub>2</sub>-induced oxidative stress activates the ERK pathway (Fig. 5). Blockage of the S1P receptor by W146 or FTY720 strongly attenuated phospho-ERK expression, similar to blockage by PD98059, implying the involvement of S1P in the activation of ERK signal by oxidants such as H<sub>2</sub>O<sub>2</sub>. In this study, we used two housekeeping genes, GAPDH for RT-PCR and  $\beta$ -actin for Western blot assay. In our laboratory setting, both RNAs were usually constant during adipogenesis of GO orbital fibroblasts and in oxidative stress condition.

Oxidative stress in adipose tissue is emerging as an important mediator of adipocyte dysfunction in obesity,<sup>35</sup> which also enhances adipogenesis in GO.<sup>23</sup> As we previously reported, adipogenesis in GO cells was upregulated by the oxidants H<sub>2</sub>O<sub>2</sub> and CSE, and that this was suppressed by the S1P1 receptor blocker W146. This suppression of adipogenesis is overtly due to the composite antiadipogenic and antioxidant effects of W146.

Our results imply that the S1P1 receptor might be a potentially valuable therapeutic target for designing new GO drugs, especially targeting adipogenesis and oxidative stress. Given that S1P receptor patterns may be complicated by the presence of multiple isoforms with opposing actions on the cell surface, receptor affinity specificity is a key element in successful S1P receptor-based therapeutic interventions.<sup>36</sup> So far, S1P1 antagonists have not reached clinical development, yet they are shown to be effective in various disease models and we are willing to suggest GO as one of new target diseases.

## CONCLUSIONS

Sphingosine-1-phosphate plays a significant role in orbital adipocyte differentiation in GO and modulation of this bioactive mediator and its receptor signaling may be a viable therapeutic target for the treatment of GO.

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